In vitro assessment of anticytosporidial efficacy and cytotoxicity of adenosine analogues using a SYBR Green real-time PCR method

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Objectives: The aims of this study were to provide a cost-effective and valuable method for evaluating drug efficacy against Cryptosporidium parvum using a quantitative SYBR Green real-time PCR (qPCR) and to assess the efficacy of adenosine analogues as drug templates.

Methods: C. parvum HNJ-1 strain growing in human ileocaecal adenocarcinoma cells was employed as an in vitro culture system. To normalize the DNA extraction efficiency, a specific plasmid was added to each sample before DNA purification; the genomic DNA of infected cells was quantified by qPCR using specific primers to confirm drug efficacy and cytotoxicity. To determine the mechanism of action, enzymatic inhibition analyses were conducted using C. parvum S-adenosyl-L-homocysteine hydrolase (CpSAHH) recombinant protein.

Results: The dose-dependent growth inhibition of C. parvum was confirmed; 50% effective concentrations of neplanocin A (NPA) and 2-fluoroadenosine (2FA) were 139 μM and 0.842 μM, respectively. Cytotoxicity evaluation showed that the 50% growth inhibition concentration of 2FA was 1.18 μM; NPA did not exhibit any cytotoxicity up to 200 μM. The screening system revealed the specific but marginal efficacy of NPA and showed 2FA to be cytotoxic. Recombinant CpSAHH inhibition analyses showed that NPA competitively inhibited CpSAHH activity (Kᵢ = 0.395 μM), whereas 2FA did not.

Conclusions: This novel qPCR system confirmed not only drug efficacy against C. parvum but also cytotoxicity to host cells. Moreover, since the SYBR Green method is cost effective, it could therefore be used in a wide variety of clinical and research-oriented applications of Cryptosporidium analysis.

Keywords: Cryptosporidium parvum, quantitative real-time PCR, in vitro culture, drug screening, adenosine analogues

Introduction

Cryptosporidiosis is a life-threatening disease mainly due to the lack of effective chemotherapy against Cryptosporidium, especially in immunocompromised individuals such as those with AIDS, and those undergoing transplantation or anticancer chemotherapy. To overcome the difficulties involved in the treatment of human cryptosporidiosis, drug-screening trials using various reagents have been conducted. Even though quantitative PCR (qPCR) assays, especially reverse transcriptase qPCRs, have been considered to be appropriate for quantifying living parasites, mRNA quantification may be affected under certain cellular conditions because of changes in expression as observed through various microarray data. Therefore, an accurate, reproducible, alternative method is required. Recently S-adenosyl-L-homocysteine hydrolase (SAHH) has been recognized as an attractive target for antiparasitic and antiviral agents. SAHH catalyses the reversible hydrolysis of S-adenosyl-L-homocysteine (SAH) to adenosine and homocysteine. SAHH thereby plays an important role in regulating the cellular levels of SAH, which is a potent feedback inhibitor of SAH-dependent methylation reactions. Moreover, SAHH is a conserved enzyme of sulphur-containing amino acid metabolism in Cryptosporidium parvum; this enzyme is a crucial metabolic enzyme and may be an attractive target for novel anticytosporidial agents.
In this study, we developed a novel method for assessing the efficacy of drugs against *C. parvum* using qPCR and assessed the anticryptosporidial efficacy and enzymatic inhibitory activity of adenosine analogues.

**Materials and methods**

**In vitro cell culture for *C. parvum***

For the collection of oocysts and the in vitro development of *C. parvum*, a previously described procedure was used with some modifications. In brief, oocysts of *C. parvum* HNU-1 strain were stored at 4°C in PBS containing penicillin and streptomycin (100 U/mL each), and were used for infection within 4 months after purification. Human ileocecal adenocarcinoma (HCT-8) cells were cultivated in 24-well plates in RPMI Medium 1640 supplemented with 10% fetal bovine serum and 4 mM L-glutamine (GluMAXTM-1) (GIBCO, Grand Island, NY, USA). After treatment with 50 mM hydrochloric acid and washing with PBS, 1.6 × 10^10 oocysts were inoculated onto cell monolayers in medium supplemented with 0.1% bile; the cells were allowed to incubate for 2 h at 37°C, and the conditions induced approximately 20% excystation (data not shown). Cell monolayers were washed twice to remove unexcysted oocysts. The medium was replaced with fresh medium with or without the tested substrates and incubated for 24 h in order to evaluate drug efficacy during the initial *C. parvum* growth before initiating the reinvasion of merozoites. As a result of the protocol, the correlation between the quantity of genomic DNA and the number of inoculated oocysts was confirmed (data not shown).

**DNA extraction**

The cell monolayers were washed twice, detached using trypsin-EDTA (GIBCO), and collected in microcentrifuge tubes by centrifugation (5 min at 5200 g). After 3 cycles of freezing and thawing, 10^9 copies of a constructed plasmid harbouring the *Cyclospora cayetanensis* 18S rRNA (Cc18SrRNA) gene were added to each collected cell sample to normalize the DNA extraction efficiency. Genomic DNA was purified using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

**qPCR assay**

The primer targets were the *C. parvum* methionine adenosyltransferase (*CpSAHH*; an anti-adenosine analogue), human β-actin (HsACTB) and Cc18SrRNA, and these were performed as three independent qPCRs. Standard plasmids containing *CpSAHH* (Sequence ID in CryptDB: CM000435; 624788–625264 bp), HsACTB (accession number: AY582799; 3274–3380 bp), and Cc18SrRNA (AF111183; 439–1074 bp) were constructed to have a dose-dependent inhibitory effect on *C. parvum* growth. As a result of the protocol, the correlation between the quantity of genomic DNA and the number of inoculated oocysts was confirmed (data not shown).

**Tested compounds**

Two adenosine analogues, neplanocin A (NPA) and 2-fluoroadenosine (2FA; Sigma-Aldrich, St Louis, MO, USA), were dissolved in 50% DMSO to prepare stock solutions.

**CpSAHH-pCold I construction and recombinant CpSAHH expression**

The subcloned CpSAHH gene (AY161083) from the pBluescript II SK(+) vector was inserted into pCold I DNA digested by EcoRV (TaKaRa). E. coli BL21 cells transformed by the constructed plasmid were harvested in LB broth with 100 μg/mL ampicillin at 37°C until the OD_{600} was between 0.4 and 0.5. His-Tag Fusion CpSAHH protein was expressed by induction with 0.5 mM IPTG at 15°C for 24 h and purified by metal affinity chromatography using His-Blind® Kits (Novagen, Merck KGaA, Darmstadt, Germany).

**Enzyme assay and kinetic analyses**

The activity of recombinant CpSAHH was assayed on the basis of a previous report. The standard reaction mixture contained SAH (6.25–200 μM) and 100 μM 5,5′-dithiobis-2-nitrobenzoate (Sigma-Aldrich) in 100 mM phosphate buffer (pH 7.2). The reaction was initiated by the addition of CpSAHH, and the increase in product was monitored spectrophotometrically at 414 nm (Labsystems iEMS Reader MF, Labsystems, Helsinki, Finland) at 37°C for 1 min. One unit was defined as the amount of enzyme that produces 1 pmol of product in 1 min. An inhibition assay was carried out by pre-incubating the standard mixture with 2FA or NPA at 37°C for 10 min and was initiated by the addition of CpSAHH. Kinetic parameters were obtained using the Michaelis–Menten equation and Lineweaver–Burk plots (SigmaPlot; Systat Software Inc., San Jose, CA, USA).

**Results**

**Anticryptosporidial efficacy of the adenosine analogues**

A qPCR assay was employed to evaluate the effect of the two adenosine analogues (Figure 1) on the growth of *C. parvum* in vitro. NPA inhibited the growth of *C. parvum* in a dose-dependent manner; the 50% effective concentration (EC_{50}) was 139 μM. No cytotoxicity was observed using NPA up to 200 μM. 2FA appeared to have a dose-dependent inhibitory effect on *C. parvum* growth (EC_{50} = 0.842 μM (Figure 1)). However, 2FA was also cytotoxic to human cells; some cells floated up in the culture medium containing high concentrations of 2FA, and the 50% inhibitory concentration (IC_{50}) to host cells was 1.18 μM. Therefore, the apparent high efficacy of 2FA was non-specific due to the suppression of host cell growth.

**Kinetic properties and inhibition analyses of CpSAHH**

The activity of recombinant CpSAHH was measured in the hydrolytic direction, and kinetic data were obtained under standard conditions. The Lineweaver–Burk plot indicated that *K_{m}* was 0.395 μM, meanwhile, 2FA (up to 100 μM) did not inhibit CpSAHH activity (data not shown).
bars indicate standard deviations.

Expression (broken line). Experiments were performed in quadruplicate. Error
expressed by the dose-response curves of NPA (filled circles) and 2FA

The growth rates of C. parvum in vitro established for evaluating drugs against
C. parvum population in endemic areas, our method can be used
for drug susceptibility or resistance evaluations at clinical sites.

Anticryptosporidial efficacy of NPA was marginal (EC 50 =
139 µM), indicating that C. parvum is relatively resistant to
adenosine analogues in contrast to Plasmodium falciparum
(EC 50 = 0.20 µM)6 and HIV (EC 50 = 0.001–0.1 µM),6 however,
only NPA competitively inhibited the activity of recombinant
CpSAHH. This fact indicates that SAHH is also a critical metabolic
step in C. parvum and may be a target of drug development.

Although further studies including in vivo evaluations are
necessary to provide novel chemotherapy, an accurate and
cost-effective SYBR Green-based qPCR assessment procedure was
established for evaluating drugs against C. parvum in vitro.
Regarding the drug screening of adenosine analogues, this
method revealed the cytotoxicity of 2FA and moderate anticryp-
tosporidial efficacy of NPA. This novel method could be used in
a wide variety of clinical and research-oriented applications of
Cryptosporidium analysis.

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Transparency declarations
None to declare.

References
1 Tzipori S, Widmer G. A hundred-year retrospective on cryptosporidiosis.


